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(54) Title: PROBIOTIC LACTIC ACID BACTERIA, UNABLE TO UTILIZE LACTOSE

(57) Abstract: The present invention pertains to a Lactic acid bacterium having probiotic properties and being deficient in using lactose as a carbon source for the preparation while still retaining the capability of translating β -galactosidase. In particular, the present invention relates to the use of such micro-organisms for the preparation of a food product containing lactose. Moreover, the present invention also relates to food compositions containing such micro-organisms.

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PROBIOTIC LACTIC ACID BACTERIA, UNABLE TO UTILISE LACTOSE

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The present invention pertains to a Lactic acid bacterium having probiotic properties and being deficient in using lactose as a carbon source for the preparation while still retaining the capability of translating β -galactosidase. In particular, the present invention relates to the use of such micro-organisms for the preparation of a food product
10 containing lactose. Moreover, the present invention also relates to food compositions containing such micro-organisms.

Micro-organisms producing lactic acid upon growth in a lactose containing medium have been known for long and are generically summarized under the group "Lactic acid
15 bacteria". This group comprises micro-organisms exhibiting quite different phenotypic traits, such as Lactococci, Bifidobacteria, Lactobacilli, Pediococci etc..

In the food industry Lactic acid bacteria have been utilized for different purposes such as for the preservation of food taking benefit of a low pH and the action of fermentation
20 products generated during the fermentative activity thereof. Also for the direct preparation of a variety of different foodstuff such as cheese, yogurt and other fermented dairy products, use is made of such bacteria.

In the recent past some strains of Lactic acid bacteria have been isolated which are
25 capable to contribute to the well-being of man and animals. Most of these strains have been found to be able to colonize the intestinal mucosa thus preventing the infestation of the individual's gut by deleterious micro-organisms and/or to be able to produce compounds that stimulate the individual's immune system.

In the food industry these strains are presently used for the preparation of so called "probiotic" products. Probiotics are considered to be viable microbial preparations which promote the individual's health by preserving the natural micro-flora in the intestine. Yet, a crucial prerequisite for their action resides in that they have to reach the guts' mucosa in an essentially proper and viable form and do not get destroyed in the upper part of the gastrointestinal tract, especially by the influence of the low pH prevailing in the stomach.

10 In WO 97/00078 such a strain, *Lactobacillus* GG (ATCC 53103), is described. This micro-organism shall be employed in a method of preventing or treating food induced hypersensitivity reactions. To this end, it is administered to a recipient together with a food material that has been subjected to a hydrolysis treatment. The *Lactobacillus* strain selected is described to exhibit a protease enzyme system, thus being capable to further
15 hydrolyze the food stuff by means of proteases secreted by the specific *Lactobacillus* strain.

In EP 0 577 903 a strain of Lactic acid bacteria is described that has the capability of displacing *Helicobacter pylori*, a micro-organism known to cause ulcer, on intestinal or
20 gastric cells. This micro-organism is proposed to be included in an ingestible carrier to be used for the therapeutic or prophylactic treatment of an ulcer associated with the action of *Helicobacter pylori*.

Further, in EP 99 104 922.2 specific strains of Lactic acid bacteria are described that
25 have the capability to prevent colonization of an individual's gut by pathogenic bacteria, that cause diarrhea, such as particular strains of *E. coli* or salmonella.

In order to provide such beneficial strains and the properties thereof, respectively, to consumers they are most often included in dairy products, such as in a yoghurt. Yet,
30 most of the probiotic Lactic acid bacteria strains known so far do not grow sufficiently

well in milk to be capable to produce some lactic acid and, hence, at least partly to acidify the milk products. However, in most cases, other lactic acid bacteria are added to the product with the probiotic strains to complete the acidification process which leads to the final fermented end-product. Rapid fermentation brings about an increased concentration of live probiotic bacteria in the final product and ensures a prolonged shelf life of the fermented product in respect to its raw material, milk.

Though, with a view to the shelf life and maintenance of the product, the lactose fermentative activity of the micro-organisms, once the fermentation process has been completed, is not desirable. On the other hand it is a desired trait that the micro-organisms should assist in the degradation of lactose in the gut, when lactose containing food material is incorporated.

Thus, there is a need in the art for avoiding the shortcomings of the state of the art and to enable the provision of probiotic milk based products, that do not acidify the product during processing and storage, but still provide active beta-galactosidase to the benefit of the consumer upon consumption of the product.

This problem has been solved by providing Lactic acid bacteria as such probiotic strains, that are deficient in using lactose as a carbon source and that are still capable to translate the β -galactosidase gene.

The present inventors have now found that when using probiotic Lactic acid bacteria that are deficient in using lactose as a carbon source, such that no lactic acid is produced, the acidification and maintaining quality of products to which such strains are added are not essentially changed.

The probiotic Lactic acid bacteria are preferably selected from the group comprising *Lactobacillus johnsonii*, *L. gasseri*, *L. acidophilus*, *L. rhamnosus*, *L. casei*, and *L. reuteri*.

In order to attain the desired traits, being unable to utilize lactose as a carbon source but still retaining the capability of translating the β -galactosidase gene, the Lactic acid bacterium preferably has a mutation in the lac-operon such that lactose may not be utilized as the carbon source. This mutation may relate to a regulatory region, or gene or to one or more of the structural genes selected from, e.g. the permease gene.

In a particular preferred embodiment the Lactic acid bacteria are CNCM - I 2369 and CNCM - I 2474, which micro-organisms have been deposited according to the Budapest Treaty with the Pasteur Institute, Paris, France on December 17th 1999 and May 9th, 2000, respectively.

It has surprisingly been recognized that by using such strains in products containing lactose, such as milk, the probiotic properties of said strains can be included in an increased number of products. It is now feasible to provide so called "value added" food products, i.e. products to which probiotic strains have been added, which probiotic strain do, however, not acidify or ferment the product. As an example for such a "value added" product common milk, supplemented with a health beneficial probiotic strain, may be mentioned. That is, the probiotic strain being deficient in using lactose as a carbon source is added to normal skimmed or unskimmed milk in a desired concentration, so that the consumer will incorporate the probiotic strain with drinking the milk, which strain will then exert its beneficial actions within the intestinal tract or the consumer.

In addition, since the strains are still able to translate the β -galactosidase gene and produce the corresponding polypeptide they may assist in the digestion of lactose in the gut, since upon lysis of the micro-organisms in the gut the cellular material inclusive the β -galactosidase will be set free and will exert its enzymatic activity.

The probiotic strain will, however, not grow in the lactose containing food product,

such as milk, yoghurt etc, such that the product may be stored in the refrigerator for the same time period as the food product without being complemented with the probiotic strain.

- 5 In order to achieve the desired goal a probiotic Lactic acid bacteria strain may be constructed, that has a mutation in the lac-operon such that lactose may not be utilized as the carbon source but still translates the β -galactosidase gene.—Preferably, the mutation lies within a structural gene, that is within the lactose operon, e.g. the permease, but it may well lie within a regulatory region of the Lac operon or a control
10 gene outside of the Lac operon.

The construction of probiotic strains deficient in utilizing lactose as the carbon source may be effected by means of recombinant technology. To this end e.g. a structural gene selected from the permease gene that has been mutated such that the gene product
15 thereof is not or is essentially not functional any more, is inserted into the location of the natural structural gene, by means of e.g. homologous recombination, with the effect that lactose may not be utilized by the micro-organism any more. Methods to genetically engineer Lactic acid bacteria are within the general knowledge of the skilled artisan.

- 20 However, such strains are preferably produced by common methods, such as mutation and selection or by simply selecting for a spontaneous mutation exerting a selective pressure on a culture of Lactic acid bacteria.

According to a particularly preferred embodiment the probiotic strains are the micro-
25 organisms deposited under the accession no. CNCM I-2369 and CNCM I-2474. These strains have been derived from the lactic acid bacterium CNCM I-1225, available from the Pasteur Institute, that has the capability to displace *Helicobacter pylori* from intestinal cells.

- 30 The products to which the strains described herein are to be added are preferably milk

derived products, since milk contains mainly lactose as the carbon source for lactic acid bacteria. To this end "value added" products, such as milk, yoghurt, curd, cheese, fermented milks, ice-cream etc. may be produced by simply adding the probiotic Lactic acid bacteria, deficient in using lactose as the carbon source to the product.

5

The following examples are for illustrative purposes only without limiting the invention thereto.

Example 1:

10 **Isolation of mutants of La1 (CNCM - 1225)**

Natural mutants of La1 were prepared in a two step process. In a first step the bacterial strain was grown in a medium containing 1% yeast extract, 1% tryptone, 1% lactose and different concentrations of penicillin (2.5 μ g/ml).

15

The ratio behind this procedure was that bacteria capable to use lactose will eventually grow and will be killed by the antibiotic. In contrast thereto, bacteria unable to use lactose will not grow and consequently not incorporate the antibiotic and stay alive.

20 In a second step surviving bacteria were plated on MRS (Difco Laboratories, MI, USA) plates containing X-gal, a substrate for β -galactosidase. This substrate is known to produce a blue coloured product upon cleavage by β -galactosidase. Thus, bacteria still capable to use lactose, should transcribe the structural polypeptides of the lac-operon, inclusive β -galactosidase, and will develop a blue colour, while mutants unable to use
25 lactose should remain white. From 200 colonies, several mutants (light blue colonies) could be isolated.

Example 2:**Stability of the mutants**

- Five colonies that remained light blue during the experiment according to example 1 (and showed a β -galactosidase activity) were further investigated for the genetic stability of the mutation. To this end, the corresponding strains were cultivated in a volume of 10 ml of MRS milk supplemented with yeast extract (0.2%). After 12 hours of incubation at 37 °C an aliquot of 0.05 ml of the culture was used to inoculate another volume of 10 ml of MRS supplemented milk as above. Each transfer of 0.1 ml to another volume was determined to be 6 generations. The stability was assessed on the basis of the natural mutants being unable to acidify the supplemented milk supplemented with yeast extract (0.2%) as above upon incubation, which can be visualized by the coagulation of casein micelles occurring at lower pH values.
- 15 The stability was assessed on the basis of the natural mutants being able to acidify the supplemented milk as above upon incubation, which can be visualized by the coagulation of casein micelles occurring at lower pH values. It could be shown that the five natural mutants did not acidify the supplemented milk even after 100 generations
- 20 The bacteria were further investigated whether they were derived from the original starter culture La1. In this respect total DNA was isolated from both the mutant and the starter strain and were digested with the restriction enzyme *MunI* under the conditions recommended by the supplier (Boehringer, Mannheim).
- 25 The DNA was transferred to an agarose gel (0.8%) and run for six hours. According to the pattern of DNA fragments generated it could be shown that the DNA of La1 and the mutants were essentially identical indicating that the isolated mutants were not contaminating bacteria.

Example 3:**Properties of La1 mutants**

- 5 Two mutants were selected for further characterization and deposited at the Institute Pasteur with the accession nos. I - 2369 and I-2474.

Bacteria were grown in milk supplemented with 0.2% yeast extract in the presence or absence of 1% glucose and incubated overnight. Growth of bacteria was determined by
10 measurement of the pH of the fermented products (table 1).

NCC	CNCM - I	PH	pH glu	OD ₄₂₀
533 (La1)	1225	4.1	4.2	.24
2655	2369	5.8	4.3	.67
2680	2474	6.0	4.4	.07

Table legend:

- Internal reference (NCC)
15 Accession no at the pasteur institute (CNCM)
pH of fermented milk (pH),
pH of fermented milk supplemented with 1% glucose (pH glu); and
 β -galactosidase activity (OD₄₂₀) of La1 and its mutants.
- 20 The two mutants are unable to grow in milk. Hence, they can not ferment lactose, and have a lactose-negative phenotype. In the presence of glucose, bacteria can grow and growth is comparable to that of the wild type La1.

The β -galactosidase activity was determined by incubating equivalent amounts of the
25 bacteria (by optical density at 600nm) in a phosphate buffer (100mm sodium phosphate pH 7.0, 1 mM MgCl₂) in the presence of ONPG (0.5 mg/ml ortho-nitrophenyl- β -D-galactopyranoside) after permeabilisation with 0.1% Triton X-100 (30 min at room temperature). Although that both mutants have a Lac-minus phenotype, they are

genetically different. NCC 2680 is unable to metabolise ONPG after permeabilisation.

This result indicates that the mutation had affected the β -galactosidase gene or a region/gene directly regulating its expression. The expressed β -galactosidase activity of strain NCC 2680 is strongly reduced. NCC2655 is still able to metabolise ONPG. In fact, the results (table 1) showed that its β -galactosidase is even more expressed than that of its mother strain, La1. In this case, β -galactosidase is still active and another gene of the lac operon or its regulation as been affected to explain the inability of strain NCC2655 to metabolise lactose.

10 Example 4:

Product prepared with La1 mutants

UHT processed milk at 3.7% fat was inoculated with the mutants of La1 (CNCM I-2369 or CNCM I-2474) (2% in a volume of frozen concentrated culture). The frozen concentrate
15 contained 5×10^9 cells/ml. The inoculated milk was mixed, distributed in 150ml plastic jars or tetra-pack type packaging and directly stored at temperature of 8°C.

A milk product was obtained which did not acidify during a shelf life of 14 days.

20 In summary from the above it may clearly be seen that the mutants described in the present specification are particularly suitable as an addition to milk products, since they impart the probiotic properties to said products, while not negatively affecting the shelf life of the resulting product.

TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

Société des Produits Nestlé S.A.
Patents Department
Avenue Nestlé 55
CH-1800 Vevey

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
l'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

NOM ET ADRESSE
DU DEPOSANT

I. IDENTIFICATION DU MICRO-ORGANISME	
Référence d'identification donnée par le DEPOSANT : NCC 2655	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE : I- 2369
II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE.	
Le micro-organisme identifié sous chiffre I était accompagné :	
<input type="checkbox"/> d'une description scientifique	
<input type="checkbox"/> d'une désignation taxonomique proposée	
(Cocher ce qui convient)	
III. RECEPTION ET ACCEPTATION	
La présente autorité de dépôt internationale a accepté le micro-organisme identifié sous chiffre I, qu'elle a reçu le 17 DECEMBRE 1999 (date du dépôt initial) ¹	
IV. RECEPTION D'UNE REQUETE EN CONVERSION	
La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial) et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)	
V. AUTORITE DE DEPOT INTERNATIONALE	
Nom : CNCM Collection Nationale de Cultures de Microorganismes Adresse : INSTITUT PASTEUR 28, Rue du Docteur Roux F-75724 PARIS CEDEX 15	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de (des) employé(s) autorisé(s) : Georges WAGENER Date : Paris, le 04 février 2000

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut
d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

CNCM
National Collection of
cultures of micro-organisms
INSTITUT PASTEUR
25, Rue du Docteur Roux
F-75724 PARIS CEDEX 15

Tel: (33-1) 45 68 82 50
Fax: (33-1) 45 68 82 36

Paris, May 09, 2000

M. Roman Vuille
vice-president
SOCIETE DES PRODUITS NESTLE S.A.
Patents Department
Avenue Nestlé 55
CH-1800 VEVÉY (Switzerland)

Via fax to numbers (0041) 21 924 28 80 / (0041) 21 785 89 25

Our Ref. (N/R): CNCM 7460.005
Obj. : Registration of a bacterium with regard to a deposit for the purposes
of the Treaty of Budapest
C/C : Mr. J.-E. GERMOND and Mrs. Aline MAMIN
Nestlé Research Center, Vers-chez-les-Blanc, Lausanne

Sir,

with the present letter, we confirm to you that we have received today with regard to an initial deposit according to the rule 6.1 of the treaty of Budapest twelve lyophilized samples relating to the here-below identified bacterium.

Your deposit project has been registered at the CNCM
with date of May 9, 2000 under the following number:

Identification reference

Registration number CNCM

NCC 2680

I-2474

If a deposit is accepted, the order number given by the CNCM is identical with the registration number and the date of the deposit is the date of the registration.

Remaining at your disposition,
I ask you to trust, Sir, in the assurance of my distinguished appreciation.

Georges WAGENER

1. Probiotic lactic acid bacterium, which is deficient in using lactose as a carbon source and which still essentially translates the β -galactosidase gene.

5

2. The Lactic acid bacterium according to claim 1, which is selected from *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Lactobacillus reuteri*.

10 3. The Lactic acid bacterium according to claim 1 or claim 2, wherein the lactic acid bacterium has a mutation such that lactose may not be utilized as the carbon source.

4. The Lactic acid bacterium according to claim 3, wherein the mutation lies in a structural gene of the Lac operon, in a gene regulating the expression of the Lac operon
15 or in a control region of the Lac operon.

5. The Lactic acid bacterium according to claim 4, wherein the mutation lies in any of the permease gene.

20 6. The Lactic acid bacterium according to claim 1, which is CNCM I-2369 and CNCM I-2474.

7. Use of a Lactic acid bacterium according to any of the preceding claims for the preparation of a food product.

25

8. The use according to claim 7, wherein the food product is a milk derived product.

9. The use according to claim 8, wherein the product is selected from milk,
30 yoghurt, curd, cheese, fermented milks, ice-cream.

10. Food product, containing a probiotic lactic acid bacterium according to any of the claims 1 to 6.

- 5 11. The food product according to claim 10, which is selected from milk, yoghurt, curd, cheese, fermented milks or ice-cream.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 01/04941

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/56 C12N1/20 A23C9/12 A23C19/032 //(C12N1/20,
C12R1:23)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 05459 A (GENENCOR INC) 31 May 1990 (1990-05-31) abstract page 12, last paragraph * see claims *	1,3-5, 7-11
X	US 5 198 351 A (SOMKUTI GEORGE A ET AL) 30 March 1993 (1993-03-30) the whole document	1,3-5, 7-11
A	LIN MEEI-YN ET AL: "Management of lactose maldigestion by consuming milk containing lactobacilli." DIGESTIVE DISEASES AND SCIENCES, vol. 43, no. 1, January 1998 (1998-01), pages 133-137, XP000960905 ISSN: 0163-2116 the whole document	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* & * document member of the same patent family

Date of the actual completion of the international search

31. August 2001

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10/09/2001

Name and mailing address of the ISA

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Authorized officer

Lejeune, R

INTERNATIONAL SEARCH REPORT

Intern. Application No.
PCT/EP 01/04941

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MUSTAPHA A ET AL: "IMPROVEMENT OF LACTOSE DIGESTION BY HUMANS FOLLOWING INGESTION OF UNFERMENTED ACIDOPHILUS MILK: INFLUENCE OF BILE SENSITIVITY, LACTOSE TRANSPORT, AND ACID TOLERANCE OF LACTOBACILLUS ACIDOPHILUS"</p> <p>JOURNAL OF DAIRY SCIENCE, US, AMERICAN DAIRY SCIENCE ASSOCIATION. CHAMPAIGN, ILLINOIS, vol. 80, no. 8, 1 August 1997 (1997-08-01), pages 1537-1545, XP000701948 ISSN: 0022-0302 the whole document</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No.

PCT/EP 01/04941

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